Human Blood Groups

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When these cells are subsequently transfected with cDNA encoding band 3, the levels of D or c and E, and of endogenously produced RhAG, are substantially increased [121,122]. This enhancing effect of band 3 expression appears to be greater on RhCcEe–RhAG complexes than on RhD–RhAG complexes [122]. The effect is reduced when K562 cells are transfected with band 3 cDNA containing the South-East Asian ovalocytosis (SAO) mutation, providing a possible explanation for reduced expression of Rh antigens on SAO red cells (see Section 10.8). Beckmann *et al.* [122] suggest that an interaction between band 3 and the Rh–RhAG complexes either enhances their translocation to the cell surface or affects their conformation in the plasma membrane.

The associations described in this section have led to proposals that the Rh polypeptides, their coprecipitating associated glycoproteins, and accessory glycoproteins exist in the red cell membrane as a protein cluster [115,123]. The nature of any association within the membrane between these structures is still a matter for conjecture. Only RhAG appears to be a requirement for expression of Rh antigens. Rh antigens are expressed normally on red cells with null phenotypes for LW, GPB (and GPA plus GPB), and Duffy. No human CD47-deficiency phenotype is known, but red cell membranes of CD47 knockout mice contained normal quantities of murine Rh and RhAG proteins [124]. There is no report of tests for Rh antigens in a band 3-deficient individual, or in band 3 knockout mice.

5.5.9 Association of the Rh complex with the membrane skeleton

Association of the Rh polypeptides, RhAG, and CD47 with the insoluble red cell membrane skeletal matrix during isolation in the non-ionic detergent Triton X100 has led to the conclusion that the Rh complex is linked to the membrane skeleton [125–128]. More direct evidence that the Rh complex is firmly linked to the membrane skeleton came from fluorescence-imaged microdeformation, which quantifies redistribution of fluorescently labelled proteins during membrane deformation induced by aspiration of the cell into a micropipette [129]. Red cells labelled with anti-D or -c Fab fragments gave results intermediate between those obtained for actin, a component of the membrane skeleton, and for band 3, a membrane glycoprotein that is firmly attached to the skeleton. The mechanism for attachment and the components of the Rh protein complex involved are unknown.

5.6 D and variants of D

D is the most immunogenic of the Rh antigens and is the most important clinically. About 80% of D– recipients of large volumes of D+ blood make anti-D and, until the introduction of immunoglobulin prophylaxis, anti-D was a common cause of severe HDN.

D+ and D– phenotypes are often referred to as Rh+ and Rh–. Between 82% and 88% of Europeans and white North Americans are D+; around 95% of black Africans are D+ [14,21]. D is a high frequency antigen in the Far East, reaching 100% in some populations. By normal blood grouping techniques, 99.7% of Hong Kong Chinese [130] and a similar proportion of Japanese [14] appear D+, but a substantial proportion of those classified as D– have a very weak D antigen called DEL (Section 5.6.4.13).

D antigen expression varies quantitatively, with a continuum of antigen strength from the greatly enhanced expression associated with D–– to weak D, the most extreme of which is DEL. Even among the common phenotypes there is readily detectable quantitative variation of D. Less D is expressed in the presence of C [131–135]; in titrations with anti-D, DcE/DcE cells give higher scores than DCe/DCe cells. Fluorescence flow cytometry with monoclonal and polyclonal anti-D demonstrated the following decreasing order of strength of D antigen: DcE/DcE > DCe/DcE > DCe/DCe > DcE/dce > DCe/dce [136–138]. Further discussion on quantitative aspects of D is found in Section 5.6.8.

5.6.1 Molecular genetics of the D polymorphism

Outside of the ABO system, the most important blood group polymorphism from a clinical viewpoint is D. D– phenotype represents a total absence of D polypeptide from the red cell membrane and, consequently, absence of all epitopes of the D antigen. This explains why d, an antigen allelic to D, has never been found.

5.6.1.1 Europeans

By Southern analysis of genomic DNA with the entire CcEe cDNA probe and with several exon specific

probes, Colin *et al.* [139] found that two highly homologous genes of identical genetic organization are present in the genome of D+ individuals, but that only one is present in D− people. Absence of the D gene from the genome of D− people was confirmed after cloning of *RHD* [57,59]. Numerous tests by a variety of techniques (see Section 5.7) have shown that homozygosity for a deletion of *RHD* is the most common cause of the D− phenotype in white people, other molecular backgrounds being rare. The deletion occurs between two 1463 bp regions of identity within the *Rh boxes* that flank *RHD* [80] (Fig. 5.4).

Tests with a technique that specifically detects intron 4 and exon 10 of *RHD* showed that all of 55 dce/dce British blood donors lacked both regions, but that discordant results were commonly associated with *dCe* and *dcE* haplotypes [76,140,141]. Of 33 dCe/dce donors, two had *RHD* intron 4 and exon 10, one had *RHD* intron 4 but not exon 10, and six had *RHD* exon 10 but not intron 4. Of three dcE/dce donors, two had *RHD* intron 4 and exon 10 and one had *RHD* intron 4 but not exon 10. Sequencing revealed that a dCe/dCe donor had a complete *RHD*, but with a nonsense mutation (Gln41stop) in exon 1 [76]. A white Australian with dCe/dce red cells had a 4-bp deletion at the 5′ end of *RHD* exon 4, which introduced a frameshift and a premature stop codon in exon 4 [142].

The frequency of the *RHD* deletion in a predominantly white English population can be estimated at about 0.39 (from data in Table 5.2).

5.6.1.2 Africans

In black Africans the most common molecular background for D− is homozygosity or hemizygosity (with an *RHD* deletion) for a complete, but inactive, *RHD* [141,143]. This inactive gene, called the *RHD* pseudogene or *RHDΨ*, contains a 37-bp sequence duplication consisting of the last 19 nucleotides of intron 3 and the first 18 nucleotides of exon 4 [143]. This duplication could generate a reading frameshift and introduce a premature translation stop codon. Alternatively, if a potential splice site at the 3′ end of the inserted intronic sequence in exon 4 were utilized, the sequence of exon 4 would remain unchanged. *RHDΨ* also has a nonsense mutation in exon 6 (Tyr269stop), which ensures that no RhD protein is present in the red cell membrane [143]. No transcript derived from *RHDΨ* was detected. *RHDΨ* is usually *in cis* with a *ce* allele of *RHCE*.

Another abnormal gene that is relatively common in D− Africans is *RHD–CE–D^s*, a hybrid gene comprising exons 1, 2, and the 3′ end of exon 3 of *RHD*, the 5′ end of exon 3 and exons 4–7 of *RHCE*, and exons 9 and 10 of *RHD* (exon 8 undetermined) [144–146]. This gene produces no D, but probably produces abnormal C. *RHD–CE–D^s* is associated with the VS+V− phenotype and the *d(C)ce^s* (r^s) haplotype (Section 5.13).

From tests on 100 black South African donors, the following frequencies of the D− alleles can be roughly estimated: *RHD* deletion, 0.10; *RHDΨ*, 0.07; *RHD–CE–D^s*, 0.04 [143,146]. Of 82 D− black Africans, 67% had *RHDΨ*, 15% had *RHD–CE–D^s*, and 18% had no *RHD*. For 54 D− African Americans the corresponding figures were 24%, 22%, and 54%, [143].

5.6.1.3 Asians

The existence of *RHD* in some D− Asians was originally indicated by the detection of *RHD* cDNA derived from erythroid progenitors from D− Japanese [58,147,148]. The molecular basis of the D− phenotype in Eastern Asia remains unclear. This is partly because of the presence of an extremely weak D antigen, called DEL, which can only be detected by very sensitive techniques, in particular adsorption and elution (Section 5.6.4.13).

In one study of 130 Japanese D− donors (DEL not found), 63% had no *RHD*, 28% (all C+) had an intact *RHD*, and two samples had probable *RHD–CE–D* hybrid genes [77]. All exons and the promoter region of the intact *RHD* genes were sequenced, but no explanation for the inactivity of these genes was found. In another study of 306 Japanese donors with an apparent D− phenotype, no *RHD* was detected in 67%; the remainder were DEL [149]. Of 87 apparent D− donors from the People's Republic of China, 60% lacked *RHD*, 25% (all C+) appeared to have an intact *RHD*, and 15% (all C+) had at least one exon of *RHD* [150]. In two studies of 230 and 204 Taiwanese, the following results were obtained: 63% and 74% with no *RHD*; 33% and 20% DEL; 4 and 6% apparent *RHD–CE–D* hybrid genes [151,152]. Where analysed, the hybrid gene comprises exons 1, 2, and 10 of *RHD* and exons 3–9 of *RHCE* [152].

5.6.2 Weak D (D^u) and partial D

Stratton [153] first coined the term D^u for a D antigen detected by only some anti-D. D^u was subdivided into 'high grade', in which the cells were directly agglutinated by some anti-D, and 'low grade', in which the D antigen could only be detected in an antiglobulin test [154,155]. D^u was shown to be inherited, the D^u allele being dominant over *d*, but recessive to normal *D* [153,155,156] (but also see Section 5.6.6). The definition of D^u evolved to become the D of those red cells that are not agglutinated by IgM anti-D, but which react with IgG anti-D in an antiglobulin test. With the introduction of modern, more potent, anti-D reagents, most red cells that would previously have been classified as D^u would not now be considered as having an abnormal D by routine testing.

D^u has been considered a purely quantitative variant of D, differing from normal D purely on the number of antigen sites per red cell [157–161]. Consequently, there can be no D^u antigen and no anti-D^u, so 'D^u' was replaced with the term 'weak D' in the 1990s [162].

The definition of weak D has often depended on the anti-D reagents and techniques used, so it is difficult to provide frequencies for the weak D phenotype. One estimate gave frequencies for weak D as 0.3% and 1.7% in white and black North London donors, respectively [163]. Molecular definitions of weak D permit more accurate determination of the frequency of weak D (Section 5.6.4.12).

If weak D (D^u) is considered a purely quantitative variant of D, then another type of D variant, now usually referred to as partial D [164,165], is a qualitative variant. Since the publication by Argall *et al.* [166] in 1953, it has been clear that rare D– individuals can make a form of alloanti-D. Many different types of partial D antigen have been identified (Section 5.6.4). The D antigen can be regarded as a mosaic of epitopes. People whose red cells lack part of the D mosaic can, when exposed to a complete D antigen, make antibody to the missing epitopes. This antibody behaves as anti-D when tested against normal (complete) D.

Weak D red cells are considered to have all epitopes of D, expressed weakly. Partial D red cells have some epitopes missing, the remainder being expressed normally. Partial weak D cells have some epitopes missing, the remainder being expressed weakly. These divisions, however, are often not distinct and are difficult to differentiate, although the ability to make alloanti-D in partial D phenotypes, but not in weak D phenotypes, is probably the most suitable definition if one is required. However, some individuals with red cell phenotypes initially regarded to be weak D have produced alloanti-D [167]. Weak D is often associated with *RHD* mutations encoding amino acid substitutions in the cytoplasmic or membrane-spanning domains of the D protein, whereas partial D is usually caused by changes in the extracellular loops [63]. However, this is not absolute and is dependent, to some extent, on the model for the conformation of the Rh proteins in the membrane used. The terms 'weak D' and 'partial D' are retained here to assist in providing some degree of order to the large number of aberrant D antigens, but there is a compelling argument in favour of scrapping the terms and replacing them with 'D variant'.

5.6.3 Partial D and the epitopes of D

The pioneering work on the subdivision of D, from which our current understanding of the immunologic profile of the D antigen is derived, was by Tippett and Sanger in the 1960s and 1970s [168,169]. They divided partial D antigens into six categories (I–VI) from the patterns of reactions between the red cells and antibodies of D+ people who made anti-D. Family studies showed all categories to be inherited. A seventh category was added later and category I is now obsolete [165,170]. Many cases of D+ people who made anti-D were also studied by Wiener and Unger [171–173]. They called the components of Rh_o (D in Wiener's language), Rh^A, Rh^B, Rh^C, and Rh^D. These subdivisions of Rh_o could not be correlated with Tippett and Sanger's categories because there was no exchange of material between the two groups and they are now obsolete.

Tippett and Sanger excluded weak D antigens from their categories and only used potent anti-D made by category members for studying the categories. The partial D antigen of a person cannot be categorized on the basis of their anti-D alone, because the immune response within a category is not consistent. Associated low frequency antigens have subdivided categories IV, V, and VI, and assist in the definition of categories III and VII, and the partial D antigens DFR, DBT, and DHAR (Table 5.5).

In the 1980s, immunological analysis of antigens was revolutionized by the introduction of monoclonal antibody technology. A plethora of monoclonal anti-D has been produced. Testing these antibodies against

Table 5.5 Variant D antigens.

Name	Molecular basis*	Exons	EC† loops	LFA‡	*RHCE*	Anti-D	No. D sites/cell¶	Ethnic group	References
DII	*RHD* A354D	7	6		Ce	Yes	3200	White	[170,174]
DIIIa	**RHD* N152T§, T201R§, F223V§	3,4,5	0	DAK	ce	Yes	12300	Black	[170,175,176]
DIIIb	**RHD–CE–D*	2	2		ce	Yes		Black	[170,177]
DIIIc	**RHD–CE–D*	3	0		Ce	Yes	22300	White	[170,179]
DIII type IV	**RHD* L62F, A137V, N152T§	2,3	0			Yes	33255	White	[167]
DIVa	**RHD* L62F, N152T§, D350H§	2,3,7	6	Go^a	ce	Yes	9300	Black	[170,180]
DIVb	**RHD–CE–D*	7–9	6	Evans	Ce, cE	Yes	4000	White, Japanese	[170,180,181]
DIV type III	**RHD–CE–D*	6–9	6		Ce		607	White	[63,167]
DIV type IV	**RHD* D350H§, G353W§, A354N§	7	6		Ce				[182]
DVa	**RHD–CE–D*	5	4	D^w	ce, Ce, cE	Yes	9400	White, Black, Japanese	[170,180,183,200]
DVI type I	**RHD–CE–D*	4,5	3,4		cE	Yes	300	White	[170,185]
DVI type II	**RHD–CE–D*	4–6	3,4	BARC	Ce	Yes	1600	White, Japanese	[170,186]
DVI type III	**RHD–CE–D*	3–6	3,4		Ce		14502	White	[187]
DVII	*RHD* L110P	2	2	TAR	Ce	Yes	3600	White	[170,188]
DAR	**RHD* T201R§, F223V§, I342T	4,5,7	0		ce	Yes		Black	[189]
DBT type I	**RHD–CE–D*	5–7	3,4	Rh32	Ce, ce	Yes	4300	White, Black, Japanese	[190,191]
DBT type II	**RHD–CE–D*	5–9	3,4,6	Rh32	Ce			Japanese	[190,192]
DCS	**RHD–CE–D*	5	4					White	[193,194]
DFR	**RHD* M169L§, M170R§, I172F§	4	3	FPTT	Ce, cE	Yes	5300	White	[180,195,213]
DFW	*RHD* H166P	4	3		Ce				[182]
DHAR	**RHCE–D–CE*	5	4	Rh33, FPTT	ce	Yes		White	[196–199]
DHK (DYO)	*RHD* E233K	5	4					Japanese	[183,184,200]
DHMi	*RHD* T283I	6	5		cE	Yes	2400	White	[201,202]
DHO	*RHD* K235T	5	4		Ce		1300	White	[203]
DHR	*RHD* R229K	5	4		cE		3800	White	[204]
DIM	*RHD* C285Y	6	5		cE		192	White	[167]
DMH	*RHD* L54P	1	1		ce	Yes		White	[205]
DNB	*RHD* G355S	7	6						[182]

Continued p. 212

Table 5.5 *Continued.*

Name	Molecular basis*	Exons	EC† loops	LFA‡	*RHCE*	Anti-D	No. D sites/cell¶	Ethnic group	References
DNU	*RHD* G353R	7	6		*Ce*		10 000	White	[174]
DOL	*RHD* M170T, F223V§	4,5	3		*ce*	Yes	4 700	Black	[194,205]
Weak D type 1	*RHD* V270G	6	0		*Ce*		1 285	White	[63,167]
Weak D type 2	*RHD* G385A	8	0		*cE*		489	White	[63,167]
Weak D type 3	*RHD* S3C	1	0		*Ce*		1 932	White	[63,167]
Weak D type 4	*RHD* T201R§, F223V§	4,5	0		*ce*		2 288	White	[63,167]
Weak D type 4.1	*RHD* W16C§, T201R§, F223V§	1,4,5	0				3 811	White	[167]
Weak D type 5	*RHD* A149D	3	0		*cE*		296	White	[63,167]
Weak D type 6	*RHD* R10Q	1	0		*Ce*		1 053	White	[63,167]
Weak D type 7	*RHD* G339E	7	0		*Ce*		2 407	White	[63,167]
Weak D type 8	*RHD* G307R	6	0		*Ce*		972	White	[63,167]
Weak D type 9	*RHD* A294P	6	0		*cE*		248	White	[63,167]
Weak D type 10	*RHD* W393R	9	0		*cE*		1 186	White	[63,167]
Weak D type 11	*RHD* M295I	6	0		*ce*		183	White	[63,167]
Weak D type 12	*RHD* G277E	6	0		*Ce*		96	White	[63,167]
Weak D type 13	*RHD* A276P	6	0		*Ce*		956	White	[63,167]
Weak D type 14	*RHD* S182T§, K198N§, T201R§	4	0		*cE*			White	[63]
Weak D type 15	*RHD* G282D	6	0		*cE*	Yes	297	White	[63,167]
Weak D type 16	*RHD* W220R	5	0		*cE*		235	White	[63,167]
Weak D type 17	*RHD* R114W	3	2**				66	White	[167]
Weak D type 18	*RHD* R7W	1	0					White	[206]
Weak D type 21	*RHD* P313L	6	0		*Ce*		5 200	White	[203]
Weak D type 22	*RHD* W408C	9	0					White	[206]
DEL	**RHD* exon 9 del	9	0		*Ce*			Taiwanese	[151]
DEL	*RHD* splice site mutation	1?	0		*Ce*			Japanese	[207]
DEL	*RHD* splice site mutation	9?	0		*Ce*			Japanese	[207]

*See Fig. 5.5.
†Predicted extracellular loops involved.
‡Associated low frequency antigens.
¶Information obtained from [167,194,208].
§Encoded by *RHCE* sequence.
** Extracellular according to one model [64], in a membrane-spanning domain according to others [62,63].

red cells with partial D antigens led to different patterns of reactions, considered to represent different epitopes of the D antigen (epD). Lomas *et al.* [209] defined seven D reaction patterns (epD1–epD7) by testing 29 monoclonal anti-D against red cells representing most of the category D antigens, and two more epitopes were added later [210–212]. Although epD6 and epD7 had been shown to differ by inhibition studies with radiolabelled antibodies [178], they could not be distinguished by agglutination techniques and were referred to as epD6/7. Eighteen of the 29 antibodies were anti-epD6/7 [209].

In 1994, Lomas *et al.* [213] gave the name DFR to a new partial D antigen, identified with the assistance of monoclonal antibodies. They refrained from making DFR category VIII because it was no longer possible to carry out all the necessary cross-testing as some of the defining anti-D made by D category members were no longer available. Since then numerous other partial D antigens have been identified (Table 5.5), creating many subsplits of the original reaction patterns. A 30-epitope model [214] was upgraded to 37 at an international workshop for monoclonal antibodies [215]. The definition of some of these reaction patterns, however, depended on the use of enzyme modified red cells. As protease modification of the D protein might affect epitope conformation, Scott [215] proposed excluding patterns that were dependent on enzyme treatment of cells from the numbering. Using only those patterns confirmed in the 1996 workshop, 24 epitopes of D could be identified [215]. The latest pattern of reactions between partial D antigens and monoclonal antibodies, shown in Table 5.6, was produced in 2001 for the fourth international workshop on monoclonal antibodies to red cell surface antigens [216]. It includes 30 epitopes, with a terminology consisting of the original epD1–epD9 (excluding epD7) [209–212], followed by numbers representing subdivisions of the epitopes (e.g. epD6.4). It is important to remember that the D epitopes really only represent reaction patterns and are not absolute. Reaction patterns may be dependent on antibody concentrations, particularly with epitopes of low affinity, so different batches of the same antibody could produce different results. Other factors, such as reaction temperature or pH, might appear to alter specificity.

5.6.4 Characteristics of D variants

Listed below and in Table 5.5 are serological and molecular characteristics of D variants. Much of the serological information is taken from a variety of publications [30,165,168–170,209,210,212,213,217] and from unpublished observations. The molecular bases for D variants involving gene rearrangements are shown in Fig. 5.6.

5.6.4.1 DII, DNU, DNB

Category II originally contained three unrelated propositi with DII travelling with *Ce* in the two families tested. The rediscovery of the original category II propositus led to the subdivision of epD3 [212]. DII is associated with an Ala354Asp substitution in the sixth extracellular loop of RhD [174]. DNU and DNB are D variants with similar epitope profiles to DII: DNU is associated with Gly353Arg [174]; DNB with Gly355Ser [182].

5.6.4.2 DIII and the DAK antigen

This is the only D category that cannot be defined by monoclonal anti-D, because category III red cells react with all monoclonal anti-D (Table 5.6). There are four subcategories of DIII. Most DIIIa and DIIIb individuals are black and the partial D travels with *ce* in families. Some members have an abnormal VS and V phenotype: their red cells react with all anti-VS, but not with all anti-V.

DIIIa cells express the low frequency antigen DAK, which is also expressed by cells with the R^N phenotype [175] (Section 5.14.1.1). DIIIa is associated with *RHD* encoding three amino acid substitutions [176] (Fig. 5.6); changes that probably represent templated microconversion events, as Thr152, Arg201, and Val223 are encoded by *RHCE*. It is possible that none of these amino acids is in an extracellular domain, though positions 152 and 223 are close to the third and fourth extracellular loops, respectively. Tests on 93 African Americans and 63 African Brazilians, by a PCR RFLP procedure, revealed frequencies for the *RHDIIIa* allele of 0.11 and 0.19, respectively [218].

Unlike most D+ red cells, DIIIb cells are G– (see Section 5.11). Two DIIIb individuals have *RHD* in which exon 2 is replaced by exon 2 from a *c* allele of *RHCE* [177] (Fig. 5.6). This results in three amino acid

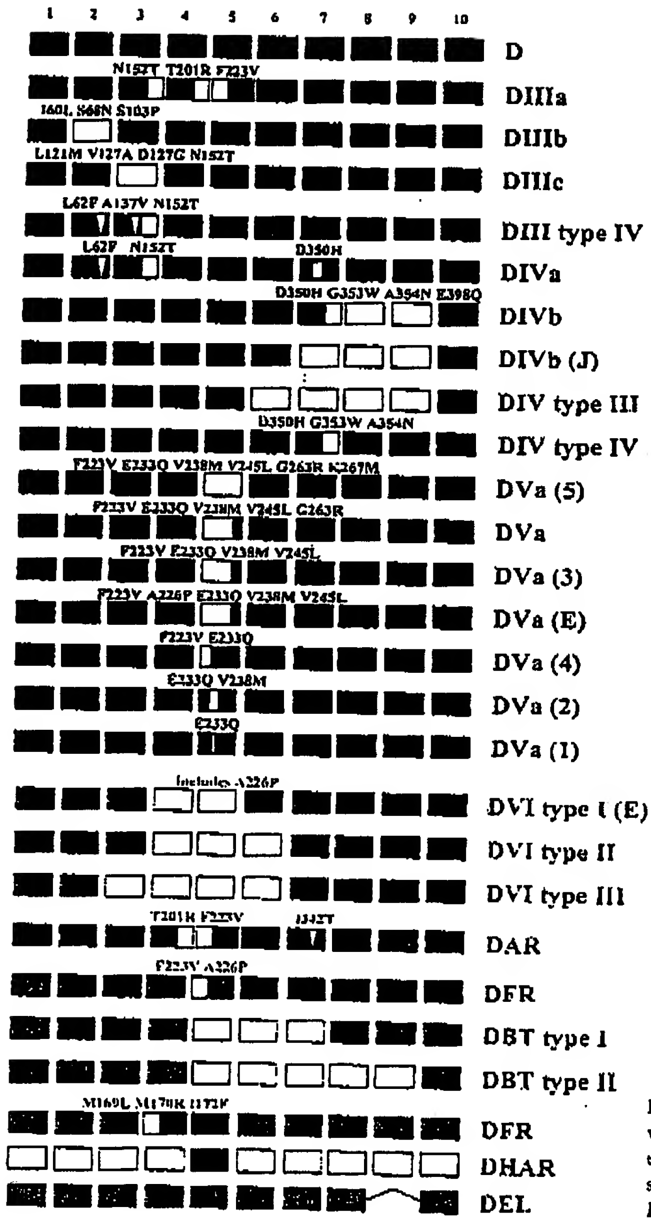

Fig. 5.6 Representation of some genes responsible for variant D antigens. ■, exons derived from *RHD*; □, exons derived from *RHCE*. ▽, untemplated amino acid substitutions. All these genes are usually paired with *RHCE*, except *DHAR*.

changes from RhD, which include a Ser103Pro substitution in the second extracellular loop. Ser103, encoded by *RHD* or the C allele of *RHCE*, is responsible for the G antigen. DIIIb has Pro103 and expresses no G.

DIIIc propositi are white and DIIIc is inherited with *Ce* in two families [179,219]. In one family, DIIIc is associated with *RHD* in which exon 3 is replaced by exon 3 from *RHCE* [179] (Fig. 5.6). It is likely that none of the four changed amino acids is in an extracellular loop, although positions 121 and 152 are close to the second and third extracellular loops, respectively.

Table 5.6 Reactions of monoclonal antibodies defining 30 epitopes of D with partial D antigens (Fourth International Workshop on Monoclonal Antibodies against Human Red Cell Surface Antigens [216]).

Anti-EpD	Partial D antigen																			
	DII	DIII	DIVa	DIVb	DVa1	DVa2	DVa3	DVa4	DVa5	DVI	DVII	DFR	DBT	DHAR	DHMi	DNB	DAR	DNU	DOL	DHK
1.1	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	+	V	V	V	−
1.2	+	+	−	−	−					−	+	−	−	−	+					
2.1	+	+	−	−	+	+	+	+	−	−	+	+	−	−	+	+	+	+	+	V
2.2	+	+	−	−	+	+	+	+	−	−	+	−	−	−	−	+	−	+	+	V
3.1	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+	V	+	+	+	+
4.1	−	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+
5.1	+	+	+	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	
5.2	+	+	+	+	+	+	−	−	−	−	+	+	−	−	+	+	+	−	+	−
5.3	+	+	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	
5.4	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+	+	+	+	V	−
5.5	+	+	+	+	−					−	+	−	−	−	−					
6.1	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
6.2	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	V
6.3	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	+	V
6.4	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	V
6.5	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+
6.6	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	V	V
6.7	+	+	+	+	+	+	−	−	−	−	+	−	−	−	V	+	+	+	+	V
6.8	+	+	+	+	+	+	+	+	+	−	+	−	−	−		+	+	+	+	V
8.1	+	+	+	+	+	+	+	+	+	−	−	−	−	−	V	+	−	−	+	V
8.2	+	+	+	+	+	+	+	+	+	−	−	−	+	−	V	+	+	+	+	+
8.3	+	+	+	+	+	+	−	−	−	−	−	−	+	−		−				
9.1	−	+	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+
10.1	+	+		−	−					−	−	−	−	−	−					
11.1	+	+	+	+	−					−	−	−	−	−	−					
12.1	+	+	+	+	+					−	+	−	+	−	−					
13.1	+	+	+	−	+	+	+	+	+	−	+	+	−	−	−	+	+	+	+	
14.1	+	+	+	−	+					−	+	+	−	−	+					
15.1	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−
16.1	+	+	+	+	+					+	+	+	+	−	+					

V, variable reactions with different antibodies.
For five types of DVa, see Fig. 5.6.

DIII type IV was added in 2000 [167]. The red cells reacted with all monoclonal anti-D and did not react with anti-D from a DIIIc individual; the anti-D from the DIII type IV patient did not react with DIIIc cells. Three amino acid substitutions encoded by *RHD* are associated with DIII type IV [167] (Fig. 5.6). Two result from untemplated mutations, whereas Asn152Thr is identical to one of the changes present in DIIIa. None is predicted to be in an extracellular loop.

5.6.4.3 DIV and the Go^a antigen (RH30)

The partial D antigen of the first DIV propositus was originally called D^{Cor} [221]. DIV appears to be elevated as judged by a few selected incomplete anti-D, which agglutinate saline suspensions of DIV red cells. DIV was initially subdivided by reactions with anti-Go^a, an antibody to a low frequency antigen [222–224]. DIVa and DIVb cells, which are Go(a+) and Go(a−), respectively, can be distinguished by monoclonal anti-D: DIVb, but not DIVa cells, lack epD4 (Table 5.6).

DIVa individuals are mostly black. DIVa travels with *ce* in most families tested, but is also present in the very rare complex DIV(C)− (Section 5.15.5). Three amino acid changes distinguish DIVa from D [180] (Fig. 5.6): two, encoded by exons 3 and 7, probably represent microconversion events; Leu62Phe (exon 2) represents an untemplated mutation. His350 (exon 7) is in the sixth extracellular loop.

Approximately 2% of African Americans are Go(a+) [225,226].

Sub-category IVb is heterogeneous; there is great variation in the strength of the partial D antigen. At least some DIVb cells have the low frequency antigen Evans (RH37) [227]. DIVb travelled with *Ce* in two families and with *cE* in three families [170]. DIVb is associated with an *RHD–CE–D* gene in which the 3′ end of exon 7, exon 9, and probably exon 8 are derived from a *RHCE* gene [180] (Fig. 5.6). All known DIVb propositi were white until four DIVb individuals were found among 5 million Japanese blood donors [228]. In Japanese, DIVb (J) results from an *RHD–CE–D* gene in which the whole of exons 7 and 9 are *RHCE*-derived [181] (Fig. 5.6).

DIV type III and type IV were defined primarily on a molecular basis. Both have an *RHD–CE–D* gene: in type III exons 6–9 have the *RHCE* sequence [63]; in type IV only part of exon 7 is exchanged [182] (Fig. 5.6).

Both types of DIVb and DIV types III and IV have Asp350His, Gly353Trp, and Ala354Asn changes in the sixth extracellular loop.

5.6.4.4 DV and the D^w antigen (RH23)

Category V has been subdivided by reactions with an antibody to a low frequency Rh antigen, anti-D^w [229]. DVa red cells are D^w+. DVa is usually produced by a $D^{Va}ce$ haplotype in black families and by $D^{Va}Ce$ in white and Japanese families; $D^{Va}cE$ is rare [170,230]. The strength of DVa antigen is very variable. The molecular background to DVa is heterogeneous, but always involves replacement of all or part of exon 5 of *RHD* by the equivalent region of *RHCE* (Fig. 5.6) [180,183,184,200,203,230]. All forms involve Glu233Gln, predicted to be in the fourth extracellular loop, and one has only Glu233Gln. All give rise to a typical DVa phenotype, so the Glu233Gln substitution must be the key to D^w expression and loss of epD1, epD5.1, and epD5.3 (Table 5.6). Red cells with the DHK phenotype, in which there is a Glu233Lys substitution, lack epD1 and some epD5 epitopes, but are D^w− [184]. One of the DVa variants, DVa(E) (Fig. 5.6), has Pro226, suggesting that the *RHCE*-derived segment of exon 5 originated from a *E* allele [231]. The red cells expressed an abnormal E antigen, despite no *E* allele of *RHCE* being present, suggesting that the *RHD–CE–D* hybrid produced some E.

Sub-category Vb contained one propositus whose red cells did not react with any anti-D from category VI individuals and were D^w− [232]. The anti-D from this propositus reacted with some DVa red cells.

5.6.4.5 DVI and the BARC antigen (RH52)

DVI has very few D epitopes and most monoclonal anti-D do not react with category VI cells (Table 5.6). A minority of anti-D from D− people react with DVI cells, which may reflect a quantitative rather than a qualitative effect [170].

Most DVI propositi are white or Japanese. DVI travels with *Ce* in most families and less commonly with *cE*. Anti-BARC, an antibody to a low frequency antigen, is a marker for the $D^{VI}Ce$ haplotype. Seventy-six of 78 $D^{VI}Ce$ samples were BARC+; all of 21 $D^{VI}cE$ samples were BARC− [170,233].

Molecular genetic analysis has revealed three types of *RHD–CE–D* encoding DVI (Fig. 5.6). The DVI type

I gene, which is always part of a $D^{VI}cE$ haplotype, has exons 4 and 5 derived from a *E* allele of *RHCE* (encoding Pro226) [185,220,234]. (The previously reported DVI gene with a deletion of exons 4–6 [186] does not exist.) DVI type II and III genes, which are part of $D^{VI}Ce$ haplotypes, have exons 4–6 and 3–6, respectively, from an *e* allele of *RHCE* (Ala226) [185–187]. DVI genes of types II and III, but not type I, produce BARC, suggesting that the presence of Ala226 in the hybrid Rh protein is important in BARC expression. Apart from the polymorphism at residue 226, all three types have the same amino acid changes from normal D in predicted extracellular loops: Met169Leu, Met170Arg, and Ile172Phe in loop 3; Glu233Gln in loop 4. Quantitative differences, in terms of numbers of D sites per cell, exist between these DVI types: type I is low with about 500 sites; type II has about 2400 sites; and type III is about normal for a DCe complex with 12 000–14 000 sites [167,187].

A unique monoclonal anti-D (LOR-15C9) binds denatured RhD on protein blots and detects the product of exon 7, binding most specifically to the region of amino acids 323–331 [235,236]. This antibody distinguishes cells with the product of a D^{VI} allele (types I–III). In addition to the M_r 33 000 RhD band, DVI cells give an M_r 21 000 band, possibly the product of an alternatively spliced transcript [235].

By screening with monoclonal antibodies, the incidence of DVI in Europe, the USA, and Australia has been estimated between 0.015% and 0.04% and represents between 5% and 16% of weak D samples [237–241]. Only one DVI was found in over 5 million Japanese donors [228].

5.6.4.6 DVII and the Tar antigen (RH40)

Characteristic of DVII is a positive reaction with anti-Tar [242–245]. Tar was associated with a *DCe* haplotype in several families [242]. Of eight DVII individuals with anti-D, two were untransfused males and in four a weak anti-D was accompanied by a strong anti-E [170,246]. Red cells of one of 1585 D+ British donors failed to react with anti-D from a DVII individual; this sample also had DVII antigen [242]. DVII results from a mutation in *RHD* exon 2 encoding Leu110Pro in the second extracellular loop of RhD [188]. Of over 60 000 German blood donors, 68 had DVII phenotype; of 33 DVII donors analysed, all had the Leu110Pro mutation [247]. An unusual *RHD* in two unrelated individuals encoded Leu110Pro and Pro103Ser (characteristic of c in *RHCE*) substitutions, and probably produced Tar and partial c (Section 5.8.4) [248].

5.6.4.7 DFR and the FPTT antigen (RH50)

Red cells with the partial D phenotype DFR react with an antibody to the low frequency Rh antigen FPTT [195,213] and give a different pattern of reactions from that seen with any of the category D red cells with anti-D from D+ and D– individuals [213]. In 23 propositi DFR was associated with a *DCe* haplotype and in two propositi with *DcE*; in two families DFR travelled with *Ce* and in one family with *cE*. Two of the DFR propositi had produced anti-D. Two of 3967 Australians, but only one of 60 000 Germans, had DFR [213,241].

DFR is associated with an *RHD–CE–D* gene in which the 5′ end of exon 4 is *RHCE*-derived [180] (Fig. 5.6). The three amino acid substitutions are located in the third extracellular loop. The molecular basis of FPTT expression is described in Section 5.17.2.

5.6.4.8 DBT and the Rh32 antigen

Rh32 is a low frequency antigen associated with the partial D antigen DBT [190] and with the $\bar{R}^N$ complex, which has normal D, but weak C and e (Section 5.14.1.1). Of eight DBT propositi tested, five had normal C and c, two had weak C (one of whom had a weak e), and one was C–c+. Three had made anti-D. The propositi were mostly of white European origin, but one was Moroccan, one Thai, and one Japanese.

In the Moroccan family, DBT is associated with *RHD–CE–D* in which the whole of exons 5–7 (and possibly 8) are *RHCE*-derived [191] (type I, Fig. 5.6). This gives rise to Glu233Gln in loop 4 and Asp350His, Gly353Trp, and Ala354Asn in loop 6. In the Japanese DBT family the *RHCE* segment of the *RHD–CE–D* gene represents exons 5–9 [192] (type II, Fig. 5.6). The product differs from that of the Moroccan DBT gene by only one amino acid change, Glu398Val, located in the cytoplasmic tail. The molecular basis of Rh32 expression is described in Section 5.17.2.

5.6.4.9 DHAR, R_0^{Har}, and the Rh33 antigen

R_0^{Har} is a rare complex consisting of a weak partial D

(DHAR), c, a very weak e, no G, and two low frequency antigens, Rh33 and FPTT [196–198] (Section 5.14.2.1). Generally, weak D antigens are most efficiently detected by an antiglobulin technique, but DHAR is different: only 7% of anti-D reacted with DHAR cells by the antiglobulin technique, although 27% reacted with enzyme-treated DHAR cells [196]. All four IgM, but only five of 24 IgG human monoclonal anti-D reacted with DHAR cells [210]. There are three reports of women with DHAR producing anti-D [198,249,250], one of which caused mild HDN [249].

The R_o^{Har} 'haplotype' differs from all others described in this section in that it comprises only one gene: there is no *RHD* or *RHCE*, but an *RHCE–D–CE* hybrid with only exon 5 representing *RHD* [199] (Fig. 5.6). Only six amino acid residues in the encoded protein are characteristic of RhD, and only one of these, Glu233, is predicted to be extracellular (loop 4). Exon 5 of *RHD* encodes Ala226, the amino acid characteristic of e, explaining the weak e expression.

5.6.4.10 DAR

DAR is a partial D antigen associated with three amino acid substitutions in RhD: two, Thr201Arg and Phe223Val, are *RHCE*-derived; Ile342Thr is untemplated [189] (Fig. 5.6). None is predicted to be extracellular, although amino acid 223 is very close to loop 4. DAR is relatively common in Africans: of 326 black South African donors, 16 (4.9%) had the *DAR* gene and five (1.5%) of these had the DAR phenotype. One DAR individual has made alloanti-D [189]. Of the 16 individuals with the *DAR* gene, all but two had an *RHCE* variant, named *ceAR*, in which most of exon 5 and the 5′ end of exon 6 is *RHD*-derived (see Section 5.13.2).

5.6.4.11 Other partial D antigens

Several other partial D antigens have been described and these are listed in Table 5.5. DCS, DHMI, DHO, DHR, DFW, DMH, DOL, DHK, and DIM are all associated with one or two amino acid substitutions in the RhD protein.

5.6.4.12 Weak D

As mentioned in Section 5.6.2, weak D is generally considered a complete D antigen, with all epitopes present, but expressed weakly. However, this is often difficult to determine because monoclonal anti-D could fail to react with weak D cells because of low avidity of the antibody, rather than complete loss of the epitope.

Initially, studies on the molecular basis of weak D revealed no changes in the sequence of *RHD* transcripts or in the *RHD* promoter region (−600 to +41) [251,252]. In 1999 Wagner *et al.* [63,167] sequenced the 10 *RHD* exons from 161 weak D samples from Germany, all with between 70 and 4000 antigen sites per cell, and found nucleotide changes encoding amino acid substitutions in all of them. Based on sequence changes, at least 21 types of weak D have been classified (Table 5.5). All the amino acid substitutions associated with weak D were in the predicted membrane-spanning or cytoplasmic domains of the RhD protein; none was extracellular [63] (although the Trp114 of type 17 would be extracellular according to a different model [61]). Weak D of types 1–3 were the most frequent, representing 70%, 18%, and 5%, respectively, of the weak D samples tested. Identification of an individual with alloanti-D and weak D type 15 red cells [167] demonstrates that the distinction between weak D and partial D is not clear, at least if production of alloanti-D remains as a definition of partial D. In fact, weak D type 4.2 is functionally identical to DAR (Section 5.6.4.10), also associated with anti-D production [189], as the *RHD* sequences differ by only a single, silent nucleotide change. Of 50 DCe/dce Australian blood donors with weak D, 76% had the type 1 mutation and 6% the type 3 mutation, whereas of 48 DcE/dce donors with weak D, 96% had the type 2 mutation [253].

The molecular background of weak remains unclear. Quantitative reverse-transcriptase PCR has shown that *RHD* genes responsible for weak D types 1, 2, and 3 have normal levels of transcription [254]. Furthermore, transfection experiments with K562 cells suggested that neither translation nor the configuration of RhD is influenced by the type 1 weak D mutation [254].

5.6.4.13 DEL (D_{el})

A very weak form of D found in the Far East is called DEL (originally D_{el}) and can only be detected reliably by adsorption–elution tests. Between 10% and 33% of Japanese and Chinese red cell samples shown to be D–

by conventional serological techniques were found to be DEL [130,149,151,152,255] yet, in some studies on Japanese donors, DEL was not found [77,79]. The DEL gene is almost exclusively *in cis* with a *Ce* allele of *RHCE*. In Taiwan, DEL was associated with a 1013-bp deletion of *RHD* extending from intron 8 to intron 9 and encompassing the whole of exon 9 [256] (Fig. 5.6). *RHD* mutations with the potential to disrupt RNA splicing were found in three Japanese with DEL phenotype. In one of the donors, homozygosity or hemizygosity for G to A in the first nucleotide of intron 1 in one donor could result in loss of exon 1 in most mRNA molecules. In the other two donors, a G1227A transition in the last nucleotide of exon 9 is a silent mutation, but might lead to abnormal splicing of exon 9.

5.6.5 A molecular approach to the structure of D epitopes

Relating the patterns of D epitope expression to the regions of D protein changed in the various partial D phenotypes has led to speculations on the structures of the D epitopes [257,258]. If the 30-epitope model (Table 5.6) is used, this can become an extremely complex exercise, especially as some epitopes appear to be dependent on a single extracellular loop, whereas others might require interactions between two or even three loops [257]. It is also important to remember that Rh epitopes are highly conformational and their expression can be affected by changes in regions of the protein other than those exposed directly to the antibody.

Liu *et al.* [259,260] approached the problem by combining the technologies of site-directed mutagenesis and the expression of cDNA constructs in K562 cells. They expressed cDNA representing the *cE* allele of *RHCE* after having changed nucleotides encoding amino acids characteristic of the third, fourth, or sixth extracellular loops of RhCcEe to those characteristic of RhD. D epitope expression was then evaluated by flow cytometry. The overall conclusions were that there are six epitope clusters, some of which are overlapping, but are located predominantly on the third, fourth, and sixth *RHD*-specific loops (Fig. 5.7, Table 5.7). None of these epitope clusters is more than 25 Å in diameter. According to this model, some anti-D recognize a 'footprint' consisting of a single extracellular loop, with others the 'footprint' could comprise two, three, or four loops.

Chang and Siegel [261] provided an alternative view of the way that monoclonal antibodies define D epitopes. They used D+ red cells to isolate Fab/phage anti-D libraries from the B cells of a producer of anti-D [262]. Information from a genetic and serological analysis of 53 unique anti-D chosen from 83 random clones demonstrated extensive genetic homology between antibodies directed against different D epitopes. Chang and Siegel [261] suggest that these antibodies would not have such similar sequences if they recognized spatially discrete and structurally unrelated regions of the D protein. They propose that antibodies to the various D epitopes bind to an 'identical footprint', which represents most or all of the extracellular expression of the protein, rather than spatially distinct epitopes. The specificity differences with partial D antigens would result therefore from conformational changes within the 'footprint'. Liu *et al.* [260], however, claim that the extracellular 'footprint' of RhD is too large to represent the whole binding site of an antibody molecule. X-ray crystallography of antibody–antigen complexes will probably be required to clarify whether D antibodies recognize a single identical 'footprint' or spatially distinct epitopes.

5.6.6 Weak D caused by the *trans* effect of C

A weak D phenotype that is not inherited in a regular fashion often occurs when the haplotype encoding the D antigen is *in trans* (on the opposite chromosome) with *dCe* or, rarely, *dCE* or *d(C)ce*^{s} (r'^{G}) [263,264]. That is, there is a *trans* effect from a haplotype encoding C, but not D. When the haplotype producing the weakly expressed D is partnered by a haplotype that encodes neither C nor D (*dce* or *dcE*) in another family member, the D is expressed normally. Some examples of DEL could result from the effect of *dCe* on a haplotype containing a weak D gene [265].

5.6.7 Elevated D antigens

Extra strong D antigens are detected by direct agglutination of red cells by incomplete anti-D, IgG antibodies that do not agglutinate red cells with normal D expression. Elevation of D associated with *D––* and related haplotypes (*Dc–*, *DC*^{w}*–*, *D··*) results from an increased number of D sites (Section 5.15). Elevation of D is also associated with some *D(C)(e)* haplotypes (Section 5.14.1) and with DIVa partial D (Section

Table 5.7 Locations of D epitopes, according to the model of Liu *et al.* [260].

D epitope	Extracellular loop required
epD2 (some)	3+4+6
epD3 (most)	6+other RhD-specific residues*
epD3 (some)	6
epD4	6+other RhD-specific residues*
epD5 (some)	3+4
epD5 (some)	3+4+6
epD5 (one)	4+6
epD6/7 (some)	3+4
epD6/7 (some)	3+4+6
epD8	1+2+3+5
epD9 (some)	6
epD9 (some)	6+other RhD-specific residues*

*Some epitopes also appear to require the presence of RhD-specific cytoplasmic and/or transmembrane residues to stabilize the configuration.

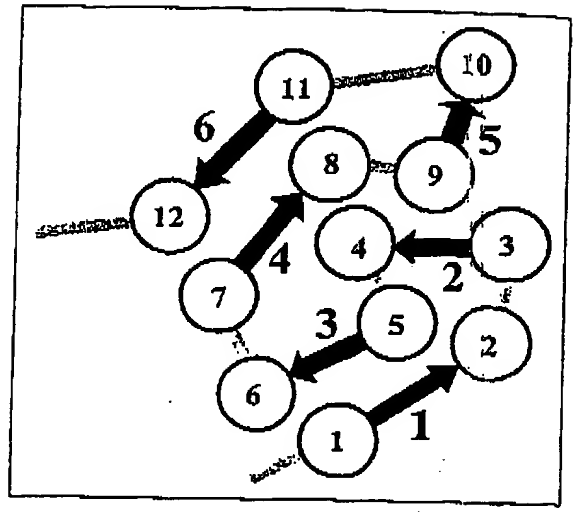

Fig. 5.7 Diagrammatic model of the RhD protein viewed from outside the membrane (i.e. from above), after [260]. Circles (numbered 1–12) represent the membrane-spanning domains, arrows (numbered 1–6) the extracellular loops, grey lines the cytoplasmic loops. See Table 5.7 for proposed location of the D epitopes.

5.6.4.3). Apparent elevation of D antigen, and of other Rh antigens, is observed in red cells with reduced sialic acid levels resulting from glycophorin A deficiency (Chapter 3).

5.6.8 Quantity of D antigen sites

The number of D sites on red cells, estimated by the use of radiolabelled anti-D and by fluorescence flow cytometry, has demonstrated that the site density differs within different phenotypes [157,167,208,266–268] (Table 5.8). As would be expected, less antigen sites were present on red cells with the weak D phenotype and the number detected was highly variable: the lowest numbers detected were about 100, the highest about 4000 [157,159,167,208]. Estimates for D site density on red cells with variant D phenotypes are shown in Table 5.5. Some partial D phenotypes, such as DIII and DIVa, have normal or greater than normal numbers of D sites per cell, whereas others, such as DVI types I and II, have very low D site density [161,167,208]. In any individual, there is a wide range in the number of D sites in each red cell [136,137].

Wagner *et al.* [167] have devised a 'Rhesus D similarity index', defined as the ratio of the 10 percentile and 90 percentile of the site densities for the various epitopes of D. Normal D antigens have an index approaching 1 as all epitopes are expressed strongly, whereas partial D antigens lacking most epitopes, such as DVI, give an index of 0. Weak D red cells give an intermediate figure. Using this index, it may be possible to predict whether a new variant D phenotype is likely to be associated with anti-D alloimmunization.

5.6.9 D variants and transfusion practice

Traditionally, red cells from donors and patients were tested with IgM anti-D by an agglutination test and those not agglutinated retested by an antiglobulin test in order to detect weak D. It is now common practice to abandon the antiglobulin phase of D testing. If two potent agglutinating anti-D are used, almost all weak D samples will be classified as D+ and only the very weakest D samples will be inaccurately labelled D–. This is generally considered acceptable for the following reasons.

1 Although weak D donations may be mistaken for D– and transfused to D– patients, red cells with weak D phenotypes do not appear to be very immunogenic (but see below).

2 Weak D patients whose red cells are typed as D– will be given D– blood with no harmful effect.

3 Weak D perinatal patients typed as D– may be given

Table 5.8 Estimated number of D antigen sites per red cell for various Rh phenotypes [167,208,266,268].

Phenotype	D sites per cell (range)
DCe/dce	9900–14600
DcE/cde	12000–19700
Dce/dce	12000–23200
DCe/DCe	14500–22800
DCe/DcE	23000–31000
DcE/DcE	15800–33300
D––/D––	110000–202000

For numbers of C, c, and e sites, see Table 5.9.

Rh immunoglobulin unnecessarily, again harmless and advantageous should they have a partial D antigen.

4 A 'false' positive result in the antiglobulin test because of a positive direct antiglobulin reaction or the presence of a contaminating antibody could result in a D– patient being typed as weak D and either receiving D+ blood or failing to receive Rh immunoglobulin.

A unit of weak D type 2 red cells was responsible for primary anti-D immunization in a D– man with no previous history of transfusion [269]. Weak D type 2 cells have the lowest D antigen density of the more common types of weak D (Table 5.5), so Flegel *et al.* [269] recommend that weak D type 2 cells should represent the threshold of detection by anti-D typing reagents and should be used in the quality assurance of these reagents. Because weak D may also be responsible for secondary immunization, it has been recommended that D– donations should be checked for weak D by an antiglobulin test before transfusion to a patient with a history of anti-D [161]. A few cases of weak D involved in severe HDN and a fatal haemolytic transfusion reaction were all reported over 40 years ago, and it is likely that these examples of weak D would be considered normal D with modern reagents [270].

The most common partial D associated with anti-D is DVI. Most DVI cells have low density of D and behave like weak D. Although some polyclonal and monoclonal anti-D reagents agglutinate DVI and most weak D cells, many monoclonal anti-D agglutinate weak D, but not DVI cells. For D typing of patients, anti-D reagents that do not react with DVI cells should be selected, as it is preferable that DVI cells be typed as D–, so that DVI patients receive D– blood.

Anti-D in women with partial D has been responsible for severe HDN [190,179,228,271–275]. Anti-D immunoglobulin should be given to partial D women during and after pregnancy, because the anti-D constituent that does not bind to the mother's own partial D cells should suppress immunization by binding to the normal D of the fetus or baby [276]. This is particularly important in DVI mothers, whose red cells lack most D epitopes.

There is little information on the immunogenicity of partial D. No case of anti-D immunization by DVI red cells is reported. DVa stimulated production of anti-D in a D– woman during her first pregnancy and resulted in mild HDN of her second baby, who also appeared to have the partial D antigen [277]. The mother had received anti-D immunoglobulin after the first pregnancy.

5.7 Predicting D phenotype from DNA

A D+ fetus of a D– woman with anti-D is at risk from HDN (Section 5.18.1.3), so it is beneficial to be able to determine the D phenotype of the fetus at a fairly early stage of pregnancy. If the fetus is D+, the pregnancy can be managed appropriately; if D–, no further action is required. Now that the molecular basis for the D– phenotype is known, it is possible to predict, with a high level of accuracy, fetal D phenotype from fetal DNA derived from amniocytes, obtained by amniocentesis, chorionic villi, and even from maternal blood.

5.7.1 Methods for testing

Several PCR-based tests have been devised to predict D phenotype. The main difficulty in formulating these tests is the design of primers that distinguish between *RHD* and *RHCE*. Initially three tests were reported, all based on detecting the presence or absence of *RHD*. One exploited a sequence that is only present in the 3′ untranslated region of *RHD* exon 10, to amplify a product from *RHD*, but not *RHCE*; a second pair of primers recognizing sequences common to exon 7 of both genes acted as a control for successful amplification [278]. Another test used sequence-specific primers to amplify products of different sizes from exons 7 of *RHD* and *RHCE* [279]. In a third method, a single pair of primers amplified across intron 4 of both genes, giving products of 600 and 1200 bp from